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13. ABSTRACT *(Maximum 200 words)*

The anti-apoptotic gene bcl-2 is frequently overexpressed in many human tumors including invasive breast cancer. Involvement of bcl-2 in cancer development was believed to result from its ability to prevent cell death (thereby increasing cell number). We previously showed that bcl-2 deregulates G_1/S checkpoint in MCF10A cells, which involves induction of cyclin D_1 associated kinase activity. Increasing evidence suggests that overexpression of cyclin D_1 contributes to the oncogenic transformation of cells *in vitro* and *in vivo* . In the present study, we examined the effects of bcl-2 overexpression on cyclin D_1 expression in human breast epithelial cells. Bcl-2 overexpression in MCF10A cells significantly induced expression of cyclin D_1 during the cell cycle as determined by an immunoblot analysis. We also show that bcl-2 induction of cyclin D_1 expression occurs at the transcriptional level as determined by an assay of cyclin D_1 promoter activity. Cyclin D_1 reporter activity was induced in a bcl-2 dependent manner in human breast carcinoma cell lines, MCF7 and BT549 as well as in MCF10A. Our previous and present studies suggest that bcl-2 may serve as an oncogene in the development of human breast cancer, which involves induction of cyclin D_1 expression.

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Introduction

The proto-oncogene bcl-2 is frequently overexpressed in many human tumors including invasive breast cancers (1, 2). *In vitro* studies clearly demonstrate that the bcl-2 gene product prevents apoptosis following a variety of stimuli including radiation, hyperthermia, growth factor withdrawal and chemotherapeutic drugs. In transgenic mice, overexpression of bcl-2 under the immunoglobulin promoter induces follicular hyperplasia, suggesting a role for bcl-2 as an oncogene. However, high levels of bcl-2 expression have shown a positive correlation in clinicopathological studies such as tumor grade, and better response to hormone treatment and chemotherapy (3). The long-term goal of our studies is to investigate the *in vivo* functions of bcl-2 in human breast cancer development. To this end, we have used MCF10A and MCF10AT models.

MCF10A cell line was established in our institute without viral or chemical intervention from mortal diploid human breast epithelial cells (4). This cell line has been utilized by many laboratories to study sequential development of differentiated or malignant states of breast epithelial cells.

Dr. Miller (consultant in this application) and his colleagues have developed an *in vivo* model system to study human breast cancer progression (5-7); Whereas MCF10A cells do not survive *in vivo* in immune deficient mice, c-Ha-ras oncogene transfected MCF10A cells (MCF10AneoT) form small nodules in Nude/Beige mice which persist for at least one year and sporadically progress to carcinomas. Cell lines have been established in cultures from lesions representing 4 successive transplant generations (designated MCF10ATG1, MCF10ATG2, MCF10ATG3 and MCF10ATG4). With each generation, cells progress to high risk lesions resembling human proliferative breast disease. Thus, the MCF10AT model provides a setting in which the steps in the conversion of the breast ductal epithelial cell to malignant disease can be studied.

Body of Report

During the 1997-1998 funding period, we have continued to investigate potential oncogenic activities of bcl-2 in breast cancer development.

Methods

Immunoblot analysis

MCF10A and bcl-2 overexpressing MCF10A clones (MCF10A bcl-2-6, MCF10A bcl-2-8, MCF10A bcl-2-30 and MCF10A bcl-2-40) were cultured as previously described (8). Cells were growth-arrested by culturing in serum-free medium for 48 hours at confluence. Cells were then treated with regular MCF10A medium containing 5% horse serum and growth factors to induce the cell cycle. At various times between 0-24 hr after serum-stimulation, whole-cell extracts were prepared using SDS lysate buffer. Protein concentrations were measured using bicinchoninic acid protein assay reagents (Pierce, Rockford, IL). Cell lysates (20 μg/lane)were denatured, subjected to SDS-PAGE analysis, and then electrophoretically transferred to a nitrocellulose membrane. Membranes were incubated with anti-cyclin D_1 (Ab2, Oncogene Research, MA), anti-bcl-2 or β-actin antibodies. Proteins were visualized using HRP-conjugated goat anti- mouse IgG (1: 3000 dilution) and chemiluminescence reagent (Dupont, Boston MA 02118). The membranes were exposed to X-ray film from 1 to 15 minutes.

Cyclin D_1 promoter activity assay

MCF10A, MCF7 and BT549 cells were transfected with -964 CD1 promoter, PCH110

(MDVlacZ), and bcl-2 expression plasmid using FuGENE6 transfection reagent (Boehringer Manheim). Fifty percent confluent cells in a 60-mm dish were transfected in 3 ml culture medium into which 500 μl FuGENE6 reaction mixture was added. The FuGENE mixture was prepared as follows; 2 μg of -964CD1 plasmid, 0.2 μg of PCH110 and increasing amounts of bcl-2 expression plasmid were mixed in 250 μl of serum free medium. The DNA solution was mixed with 250 μl of serum free-medium which contains 50 μl FuGENE6 reagent. The FuGENE6 mixture was incubated at room temperature for 30 minutes before transfection. Cells were harvested after 48 hours of transfection with 1X reporter lysis buffer. After mixing 20 μl of cell lysate and 100 μl luciferase substrate, the luciferase activity was measured using a luminometer. The β-galactosidase activity was measured by a chemiluminescent reporter assay (Tropix) to normalize transfection efficiencies. Protein concentration of the cell lysate was measured using the BCA reagent (Pierce).

Results

We previously showed that bcl-2 deregulates G_1/S check point through modulation of cyclin D_1-associated kinase activity. In the present study, we examined whether bcl-2 regulates cyclin D_1 expression. When the levels of cyclin D_1 expression were examined in growing cells of MCF10A and bcl-2 overexpressing MCF10A cells, it appeared that bcl-2 induced expression of cyclin D_1 (Fig 1). To exclude the possibility that the differences in the levels of cyclin D_1 reflect the differences in the cell cycle distribution between the control and bcl-2 overexpressing cells, we examined the effects of bcl-2 overexpression on cyclin D_1 expression during the cell cycle. The control and bcl-2 overexpressing MCF10A cells were synchronized at G_0 by culturing in serum-free medium for 48 hours and the cell cycle was induced by culturing them in complete MCF10A culture medium. At various times between 0-24 hr after cell cycle induction, whole-cell extracts were prepared and the levels of cyclin D_1 were determined by immunoblot analysis. As shown in Fig. 2, the levels of cyclin D_1 expression were significantly higher throughout the cell cycle in bcl-2 overexpressing cells than in the control cells.

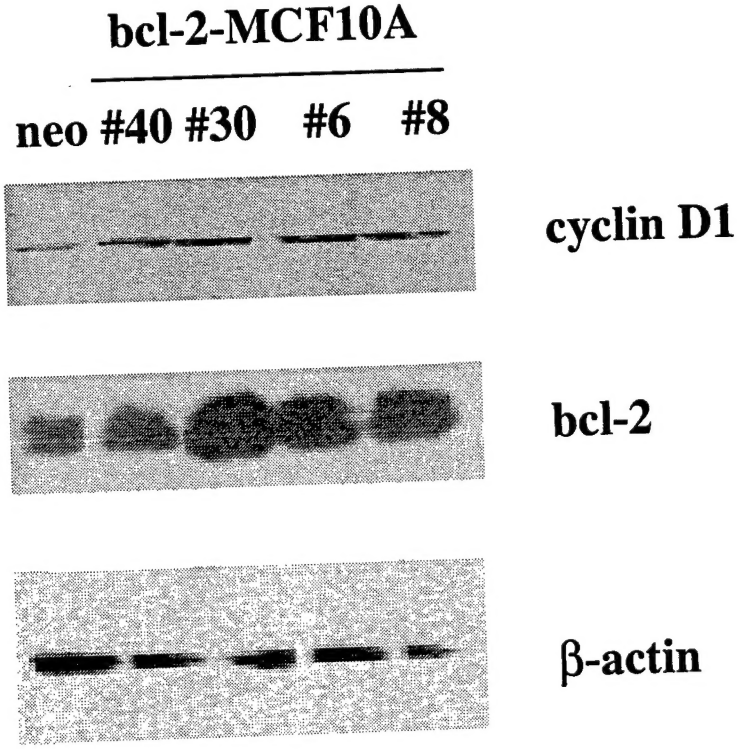


Fig. 1. Bcl-2 induces cyclin D_1 expression in MCF10A cells. The levels of cyclin D_1 (top panel) and bcl-2 (middle panel) proteins were determined by immunoblot analyses. Protein samples were prepared from neo-resistant vector transfected MCF10A (lane 1) and bcl-2 overexpressing MCF10A clones (lanes 2-5). To confirm the amount and quality of proteins loaded in each lane, the identical blot was probed with anti-β-actin antibody (bottom panel).

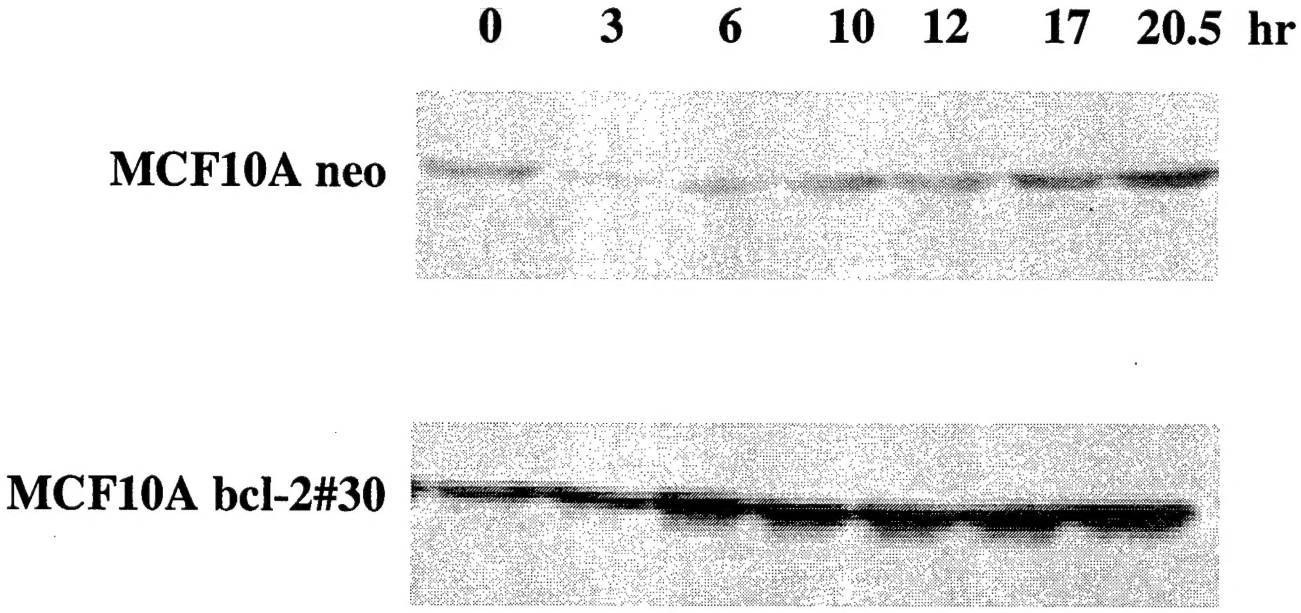
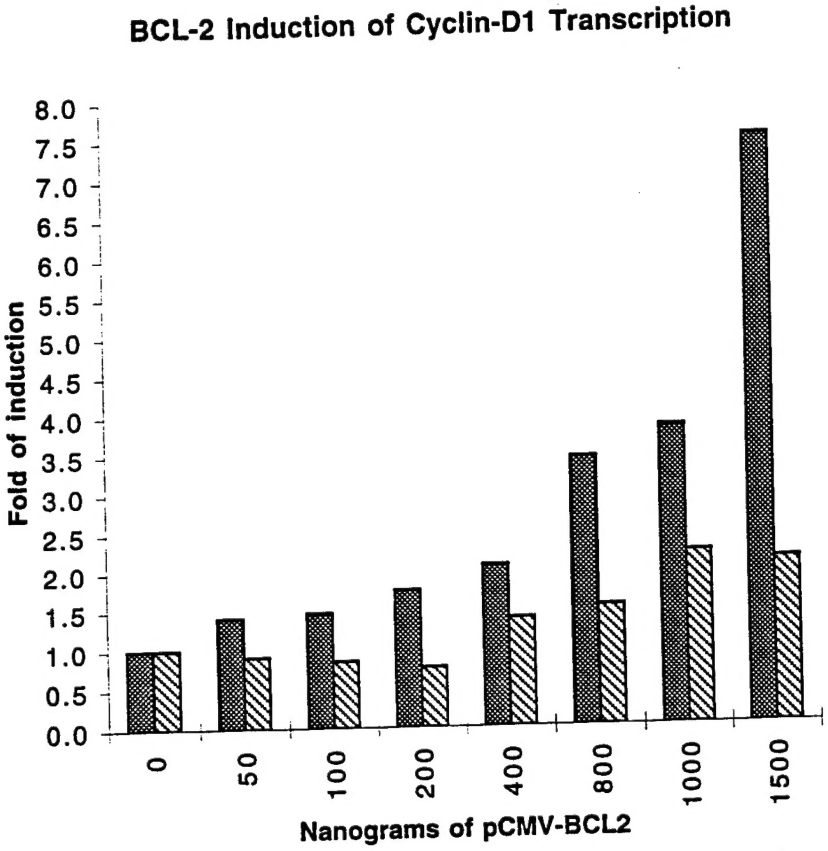


Fig. 2. Bcl-2 induces cyclin D1 expression during the cell cycle in MCF10A cells. Confluent control (top panel) and bcl-2 overexpressing MCF10A clone 30 (bottom panel) were cultured in serum free DMEM/F12 medium for 48 hours. At various time points after serum treatment, cells were lysed using SDS sample buffer. Levels of cyclin D_1 protein were determined by immunoblot analyses.

We then examined whether bcl-2 induces transcription of cyclin D_1 in MCF10A cells. The effects of bcl-2 on cyclin D_1 promoter activity were determined by cotransfection experiments using -964CD1LUC plasmid (containing the human cyclin D_1 promoter linked to the luciferase reporter provided by Dr. Pestell, see ref. 12) and bcl-2 expression vector. As shown in Fig 3A, cyclin D_1 reporter activity was induced in a bcl-2 dependent manner. It was examined whether bcl-2 induction of cyclin D_1 transcription occurs in other human breast epithelial cells. To this end, we used human breast carcinoma cell lines, MCF7 and BT549. As shown in Fig 3A and B, bcl-2 effectively induced promoter activities of cyclin in these cell lines. These results show that bcl-2 induces expression of cyclin D_1 at the transcriptional level in human breast epithelial cells.

A.


BCL-2 Induction of Cyclin-D1 Transcription
Fold of induction
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
50
100
200
400
800
1000
1500
Nanograms of pCMV-BCL2

B.

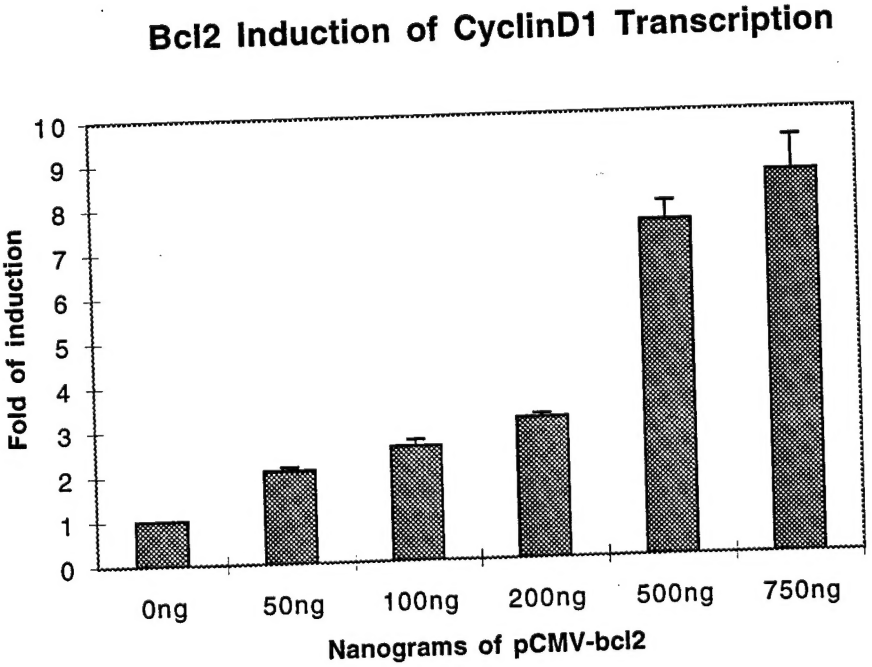

Bcl2 Induction of CyclinD1 Transcription
Fold of induction
10
9
8
7
6
5
4
3
2
1
0
0ng
50ng
100ng
200ng
500ng
750ng
Nanograms of pCMV-bcl2

Fig. 3. Bcl-2 induces cyclin D_1 promoter activity in human breast epithelial cells. A. -964CD1LUC reporter was transfected with increasing amounts of bcl-2 expression vector into MCF10A (hatched bar in panel A), BT549 (dotted bar in panel A) and MCF 7 (panel B). All experiments were performed in triplicates and the folds of induction represent the luciferase activity/LacZ activity/mg protein.

We are currently investigating the regions of the cyclin D_1 promoter responsible for transcriptional activation by bcl-2 overexpression using control and bcl-2 overexpressing cells. Our preliminary study showed that mutation of the AP1 binding site in the cyclin D_1 promoter does not have any effect on bcl-2 activation of cyclin D_1 promoter activity. However, deletion of the SP-1 binding site in -163 significantly reduced bcl-2 activation of cyclin D_1 promoter activity (data not shown). Further studies are in demand to determine the mechanisms by which bcl-2 activates cyclin D_1 promoter activity.

Conclusion

Increasing evidence suggests that overexpression of cyclin D_1 contributes to the oncogenic transformation of cells *in vitro* and *in vivo* (9-11). Involvement of bcl-2 in cancer development was believed to result from its ability to prevent cell death (thereby increasing cell number). However, our previous and present studies suggest that bcl-2 may serve as an oncogene in the development of human breast cancer, which involves induction of cyclin D_1 expression. We now wish to determine the *in vivo* oncogenic role of bcl-2 using bcl-2 overexpressing MCF10ATG3B (we previously showed that bcl-2 overexpression in MCF10ATG3B induces a transformed phenotype as determined by a soft agar assay). Since the Career Development Award does not support any research expense, it is difficult to perform the proposed study which includes animal experiments.

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